AD_____

Award Number: DAMD17-99-1-9321

TITLE: Oxidized DNA Bases in Breast Cancer

PRINCIPAL INVESTIGATOR: Mehul Desai, Ph.D.
Krystyna Frenkel, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine
New York, New York 10016

REPORT DATE: August 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual Summary (1 Aug 99 - 31 Jul 02)

4. TITLE AND SUBTITLE
Oxidized DNA Bases in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9321

6. AUTHOR(S) :
Mehul Desai, Ph.D.
Krystyna Frenkel, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York University School of Medicine
New York, New York 10016

E-MAIL:
mehul.desai@med.nyu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING

20030602 007

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Recently more studies have correlated the elevated 5-hydroxymethyl-2'-deoxyuridine (HMdU - an oxidized DNA base) levels with an increased risk of cancer when compared to controls. HMdU has been proposed to be a biomarker for breast cancer risk. Although methods are available, but requires larger sample sizes (between 50-100 μg DNA), which limits its application for HMdU quantitation in relatively smaller sample size. We have developed a sensitive method to detect and quantitate HMdU using fluorescent coumarin derivative to label HMdU and other nucleosides. The limit of quantitation for this method is linear in the range of 0.2 – 5 pmol. This method is applied to measure HMdU in small samples of calf thymus DNA (10 μg – 25 μg) and WBC DNA. To quantitate HMdU, cellular DNA is enzymatically digested to nucleosides, which are well separated by reverse-phase HPLC-1. HMdU-containing fractions are concentrated, and re-purified using HPLC and subjected to coumarin post-labeling and quantitated using normal phase HPLC-2 with fluorescence detection. The ratio of HMdU/10^5N (total 2'-deoxyribonucleosides) from 6 individually processed and analyzed 25 μg calf thymus DNA samples was 1.65±0.21(SE). Ratios of HMdU/10^5N in WBC and HEPG-2 cells were found to be 4.6 and 1.7 respectively.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
HmdU post-labeling, fluorescent labeling, HmdU, breast cancer	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

1. Introduction:

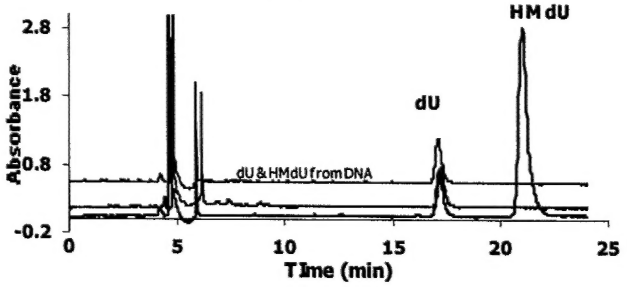
Increasing evidence implicates oxidative DNA damage as playing an important role in the development of breast as well as other types of cancer. Our hypothesis is that oxidative DNA damage begins much earlier than the clinical manifestations of breast cancer. Recently more studies have correlated the elevated 5-hydroxymethyl-2'-deoxyuridine (HMdU - an oxidized DNA base) levels with an increased risk of cancer when compared to controls. HMdU has been proposed to be a biomarker for breast cancer risk. Our laboratory has shown that sera of healthy women with a family history of breast cancer and women apparently healthy that have been diagnosed with breast cancer several years after blood donation had elevated amounts of anti-HMdU antibodies. This suggests that in the early stages of cancer development cells are under oxidative stress, which is manifested by significant HMdU formation. Analytical methods are available for the detection and quantitation of HMdU, such as ^{32}P- and ^{3}H- post-labeling, which are very sensitive, but these methods pose the problem of radioactive disposal when applied to larger groups of samples. HMdU has been also quantitated reliably using GC/MS. This technique can give accurate results but requires specialized, expensive instrumentation and qualified personnel to operate, which not too many labs can afford. In addition, GC/MS technique for HMdU quantitation requires larger sample sizes (between 50-100 μg DNA), which limits its application and does not allow to quantitate HMdU in smaller sample. To test our hypothesis we have developed a sensitive method to detect and quantitate HMdU using fluorescent coumarin derivative to label HMdU and other nucleosides. This method was applied to measure HMdU in a small sample (10 μg -25 μg DNA) of calf thymus DNA and also applied to quantititate HMdU in DNA of white blood cells.

2. Studies and Results:

In previous reports we have described the fluorescent post-labeling of HMdU, using 7-dimethyl amino coumarin-3-acetic acid (DMACA) in the presence of 4-pyrolidinopyridine (Ppy) and dicyclohexyl carbodiimide. We have discussed the labeling sites and analysis of the fluorescent products of 2'-deoxyribonucleosides using normal phase HPLC. The detection limits of the purified fluorescent products were found to be 0.01 fmol. The fluorescence labeling reaction was optimized using standard HMdU and limits of the detection and quantitation were obtained by using progressively smaller amounts of HMdU for the labeling reaction. We have also described the use of 2'-deoxyuridine (dU) as a reporter molecule; a known amount of dU was added to the HMdU fluorescent labeling reaction, which would undergo the same labeling reaction as HMdU and would account for losses during the reaction, pre-purification and analysis. This fluorescent labeling method was linear in the range of 0.2 –5.0 pmol, levels expected to occur in cellular DNA.

In the last year we tried to solve the problem that has arisen during the fluorescent post-labeling of HMdU isolated from DNA, as an unidentified fluorescent impurity was co-eluting with fluorescently-labeled HMdU products. To resolve this interference we have included one more purification step for HMdU obtained from DNA before fluorescent post-labeling reaction. The HMdU fractions obtained from separation of 2'deoxyribo – nucleosides (HPLC-1) were further purified

Fig. 1 :Separation of dU & HMdU on Normal Phsae HPLC (HPLC-1N)

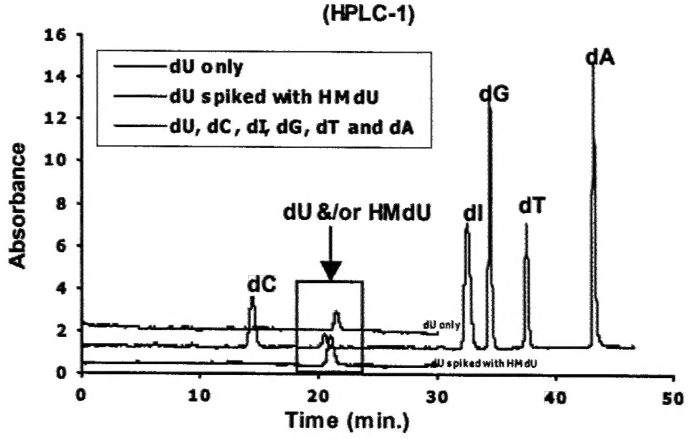


HPLC Parameters: *Column: Beckman Ultrasphere Silica (5μm, 10 mm i.d. X 250 mm) with guard column Ultrasphere Silica (5μm, 4.6 mm i.d.X 45 mm) Mobile Phase : 7% Mehanol / 43 % Methylene chloride/ 50 % n- Hexane Flow rate: 3 ml / min.; Detection :UV @ 254 nm*

using normal phase HPLC (HPLC-1N). Under the HPLC parameters for normal phase HPLC (HPLC parameters are given in Fig. 1) HMdU elutes after dU. The post-labeling of HMdU fraction showed that the original DNA derived impurity was removed which allowed for a reliable quantitation of HMdU.

To trace the losses during the whole process of the separation of HMdU from DNA to the point of getting HMdU in pure form before fluorescent labeling reaction, we have added 1 nmol dU (which is detectable by UV detector at 254 nm) to the hydrolyzed DNA (from calf thymus, salmon testes or isolated from WBC). Since the HMdU isolated from DNA is undetectable by UV, addition of dU allowed us to locate HMdU during HPLC purification. We have improved the previously used HPLC-1 method in such a way that dU and HMdU elute together but are separated from other 2'-deoxy-ribonucleosides (Fig. 2). The fraction containing both dU and HMdU are collected, which allows us to isolate all of the HMdU, dried and re-purified using HPLC-1N, dried again and subjected to the fluorescent labeling reaction.

Fig. 2 : HPLC Profile for Separation of 2'-Deoxyribonucleosides (HPLC-1)

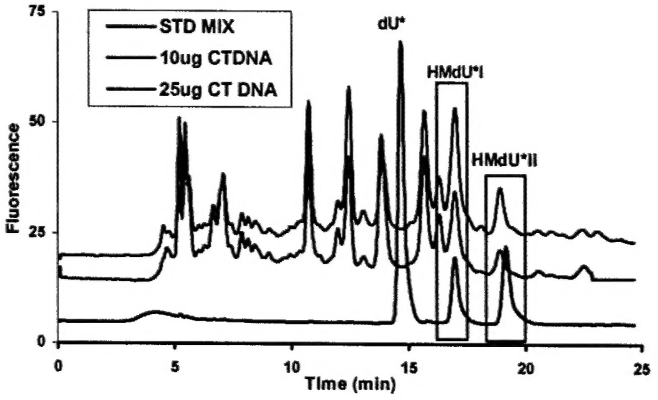


HPLC Parameters:
Column: Beckman Ultrasphere C_{18} (5µm, 10 mm i.d. X 250 mm) with guard column Ultrasphere C_{18} (5µm, 4.6 mm i.d.X 45 mm); Mobile Phase : 5mM ammonium formate / Acetonitrile; Flow rate: 2ml / min.; Detection : UV @ 254 nm;

Fluorescent Post-labeling of HMdU isolated from DNA :

The purified HMdU fraction obtained from normal phase HPLC was dried on Speed Vac and subjected to fluorescent post-labeling reaction (the optimized conditions for HMdU fluorescent labeling reaction were described in our earlier reports). The analysis of HMdU-coumarin labeled products after the pre-purification on silica SEP-PAK showed that the most of the impurities were removed during the 2nd purification step (HPLC-1N) and fluorescent products of HMdU obtained from the DNA samples were base-line separated from the impurity peak. The HPLC profile of the fluorescent products of HMdU isolated from calf thymus DNA is shown in Figure 3.

Fig. 3 : HPLC Profile (HPLC-2) for Fluorescent Post-Labeling of HMdU isolated from calf thymus DNA



HPLC Parameters: *Column: Beckman Ultrasphere Silica (5µm, 10 mm i.d. X 250 mm) with guard column Ultrasphere Silica (5µm, 4.6 mm i.d.X 45 mm) Mobile Phase : 5mM ammonium formate / Acetonitrile; Flow rate: 2ml / min.; Detection : Fluorescence , λ_{EM} = 380 nm and λ_{EM} = 460 nm*

As discussed earlier, we have used dU as a reporter molecule for the fluorescent post-labeling reaction, which would undergo fluorescent labeling reaction along with HMdU and assist in the accounting for the losses during reaction and purification. Since we have included dU to locate HMdU

on HPLC-1 chromatography run and in the 2nd purification step (HPLC-1N), we had to discontinue the use of dU as a reporter molecule.

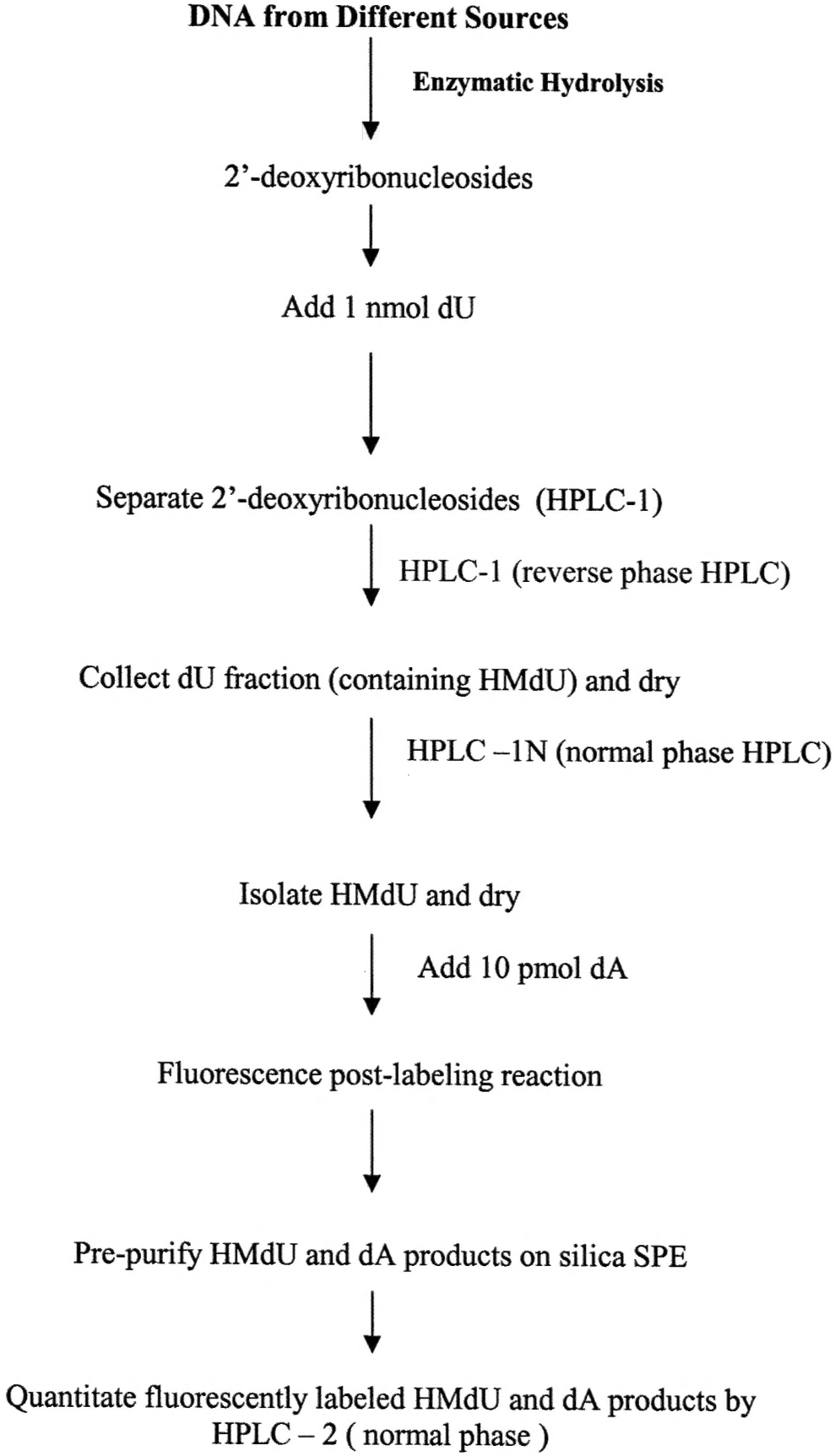
Analysis of DNA samples :

The developed method was applied to the quantitation of HMdU in DNA from different sources (calf thymus, salmon testes and DNA isolated from the WBC). The DNA was enzymatically hydrolyzd, 1 nmol dU was added before HPLC-1 analysis. The fluorescent labeling procedure was followed as shown in a flow chart shown on the next page of this report. Data were analyzed with respect to the yield of HMdU *I and/or II based on 5 pmol HMdU fluorescent labeling reactions carried out in parallel with the HMdU fluorescent post-labeling reaction from DNA samples. The data for HMdU quantification from DNA is calculated as HMdU/10^5 dT and HMdU/10^5 total nucleosides (N). Ratios of HMdU/10^5N in WBC and HEPG-2 cells were found to be 4.6 and 1.7 respectively. Six 25 µg calf thymus DNA samples were taken for analysis. As this table shows, this fluorescent post-labeling method is reproducible, since these DNA samples were independently processed and analyzed.

DNA Sample 25 µg	dT (n mol)	Total 2'deoxyribo-nucleosides (N)	Ratio HmdU/10^5 dT	Ratio HMdU/10^5 N
Calf thymus	13.28	51.49	3.7	0.9
	13.03	51.75	6.5	1.6
	13.96	52.41	8.9	2.4
	13.92	52.20	4.8	1.3
	13.56	52.84	7.2	1.8
	13.74	52.02	7.1	1.9
Mean	13.58	52.12	6.37	1.65
SE	0.15	0.20	0.76	0.21
COV (%)	1.11	0.38	11.90	12.78

We think that the use of the reporter molecule is still important, because it would allow us to monitor the fluorescent labeling reaction. The losses during the fluorescent labeling reaction can be compensated following the yield of the fluorescent product of the reporter molecule. The analysis of the data for HMdU quantitation from calf thymus DNA suggests that the incorporation of the reporter molecule during the fluorescent labeling reaction would be critical for even more accurate quantification of HMdU in DNA samples. After exploring different 2'-deoxyribonucleosides, dA was selected as a new reporter molecule, since the fluorescent product from dA elutes after fluorescent HMdU products. We intend to add 10 pmol of dA to fluorescent post-labeling reaction. We are still in the process of modifying existing HPLC-2 elution conditions for better resolution of HMdU*I & II products in the presence of fluorescently labeled dA (dA*), because dA* is retained for too long on the column under existing HPLC-2 parameters.

Following the changes made during the course of time, finally we are able to put together the fluorescence post labeling method for the detection and quantitation of HMdU present in DNA samples in a reliable and reproducible manner. The Schematic of the Method is summarized as follows:


DNA from Different Sources
Enzymatic Hydrolysis
2'-deoxyribonucleosides
Add 1 nmol dU
Separate 2'-deoxyribonucleosides (HPLC-1)
HPLC-1 (reverse phase HPLC)
Collect dU fraction (containing HMdU) and dry
HPLC –1N (normal phase HPLC)
Isolate HMdU and dry
Add 10 pmol dA
Fluorescence post-labeling reaction
Pre-purify HMdU and dA products on silica SPE
Quantitate fluorescently labeled HMdU and dA products by
HPLC – 2 (normal phase)

3. Significance

The most significant finding stemming from the studies done during this time period is that, the fluorescent post-labeling method was developed and validated with a linearity being in the range of 0.2 – 5 pmol for detection and quantitation of HMdU. This method was applied to quantitate HMdU isolated form DNA from various sources. This fluorescent labeling technique can be applied to fluorescently label other 2'-deoxyribonucleosides as well.

4. Plan

We intend to analyze the samples, as suggested in the proposal, obtained from Women Health Study (WHS) at NYUSM. The DNA has been isolated from the blood clots and is stored at –80°C.

Research Accomplishments to date:

- Fluorescence post-labeling of HMdU was accomplished by acylation with 7-dimethylamino coumarin-4-acetic acid (DMACA).

- Post-labeled HMdU, dU & dT product showed that the labeling occurs on the primary hydroxyl (-CH_2OH) group as compared to secondary hydroxy group.

- Normal phase HPLC method was developed for the separation of post-labeled HMdU products; this method can detect and quantitate HMdU in femtomole range

- HMdU post-labeling method was optimized and limit of detection (LOD) and limit of qunatitation (LOQ) for the HMdU fluorescent post-labeling method were obtained; quantitation is linear in the range of 0.2 – 5 pmol

- Isolation of HMdU and its purification were monitored by including 1 nmol dU to DNA hydroxylate

- Fluorescent post-labeling method was applied to quantiate HMdU from DNA samples and the results from 6 individually processed and analyzed 25 μg calf thymus DNA shows the ratio of HMdU/10^5N (total 2'-deoxyribonucleosides) as 1.65 ± 0.21(SE). Ratios of HMdU/10^5N (total 2'-deoxyribonucleosides) in WBC and HEPG-2 cells were found to be 4.6 and 1.7 respectively.

Repotable Outcome: N/A

Conclusions:

We have developed a sensitive fluorescence post-labeling method for quantitation of HMdU, an oxidized DNA base, which will be utilized for analysis of DNA isolated from human WBC, as a measure of oxidative DNA damage. The HMdU-post labeling was accomplished using 7-dimethylamino-coumarin-3-acetic acid in the presence of catalysts. The method was optimized by varying all reagents and parameters involved in the reaction. The limit of detection and limit of quantitation for this method is in the range of 0.2 – 5 pmol. This method was utilized for the analysis of HMdU isolated from DNA form calf thymus DNA. Results from 6 individually processed and analyzed 25 μg calf thymus DNA show ratio of HMdU/10^5N (total 2'-deoxyribonucleosides) being 1.65 ± 0.21(SE). WBC and HEPG-2 cells displayed HMdU/10^5N (total 2'-deoxyribo-nucleosides) ratios of 4.6 and 1.7 respectively. We are working on use of another reporter molecule that would allow us to account for the losses during the fluorescent labeling reaction. This fluorescence post-labeling technique can be used for sensitive detection of other modified nucleosides.